IPM Final Report

Title: **Employing foliar endophytes as biocontrol agents**

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Abstract:

Foliar fungal endophytes are microscopic fungi present in all plant species. These organisms can reduce pest feeding on plant hosts through the production of unpalatable chemicals. Endophyte-host effects have lead to the employment of fungal endophytes as biocontrols in grasses by successfully reducing pest loads while increasing host growth. However, utilization of endophytes in woody plants is in the nascent stages. Research is needed to identify which endophytes are present and how they reduce pest damage on important ornamental and horticultural plants. The proposed research is foundational for the effective use of fungal endophytes in integrated pest management.

Background and Justification:

Much of the research exploring the ecological consequences of foliar endophyte-plant relations have been limited to *Epichloë/ Neotyphodium* endophytes infecting agronomic and horticultural grasses. In these hosts *Epichloë* and *Neotyphodium* species increase host resistance to numerous plant pathogens and herbivores (Latch 1993, White et al. 1993, Gange 1996, Bultman et al. 2006, Sullivan et al. 2007, Kuldau and Bacon 2008, Tian et al. 2008), via production of alkaloids or mycotoxins (Ball et al. 1995, Frey et al. 1997, Arnold et al. 2003, Dingle and McGee 2003, Seto et al. 2007, Mejía et al. 2008). As such, particular endophytic compounds have been isolated for development of new pesticides (Ondeyka et al. 1997, Strobel 2002, 2006) and unique endophyte-host combinations have been and continue to be developed to increase forage and horticultural plant production (Siegel et al. 1987, Bouton et al. 2002, West and Piper 2008).

In woody plant species endophytes have been comparatively less well researched but initial results support their importance and potential application similar to those of *Epichloë/ Neotyphodium* endophytes (Kumar et al. 2008). For example, endophytes infecting woody species reduce survival of a suite invertebrate herbivores and retard fungal and bacterial pathogens (Sneh 1998, Arnold et al. 2003, Dingle and McGee 2003, Mejía et al. 2008). In total this research has lead to the suggested use of endophytes as an integrated pest management (IPM) approach. This is due to the expectation that fungal symbionts can reduce pesticide, fungicide, and fertilizer usage in woody crops similar to their use in grasses (Brimner and Boland 2003, Wicklow et al. 2005, Kumar et al. 2008). By integrating endophytes into IPM strategies the use of chemicals toxic to humans and the larger environment can be reduced. This type of research is timely because though little is known about the effects of endophytes on woody plants there is data to support the effective use of these symbionts in the development of IPM protocols. Our project proposes to capitalize on the recognized improvement of growth and herbivory tolerance in endophyte infected plants and target our results to the improvement of woody ornamental crops.

Objectives:

1. Remove systemic fungal endophytes from a model, woody, host plant to create endophyte infected (E+) and endophyte-free (E-) lines for experimental treatments.
2. Determine if endophytic infection alters host response to a generalist invertebrate herbivore and fungal pathogen.
3. Quantify changes in host biomass production as a general response by comparing E+ and E- controls.
4. Project Evaluation – presentation at the Green Industry Conference will provide opportunities to discuss application with growers and to get feedback from growers about how IPM application might impact them.

Procedures:

Initial research will target *Populus* sp*.* a fast growing, model species that is easily propagated (Lemus and Lal 2005**,** Das et al. 2009) and for which a rich data set exists, e.g. genetic maps, protein and metabolic expression profile. While we recognize *Populus* is only marginal in terms of importance in the ornamental industry, the enormous library of information on *Populus* will allow more rapid achievement of our proposed objectives. This will reduce the resources, including time, required to establish a primary outcome and will facilitate the adoption of our methods to other important ornamental woody species.

Populus clones will be hydroponically grown to remove fungal endophytes. Plants produced from the cloned, hydroponically grown tissues will be used to create two endophyte groups E+ and E-. Uninfected plants will be produced hydroponically as per Faeth and Sullivan (2003). All plants will be then grown in a soilless mix with a single application of a full-spectrum nutrient and watered as needed.

UPDATE:

Due to loss of all Populus trees resulting from a combination of greenhouse insect outbreaks and photoxic response following multiple insecticide applications we have modified the experiment by working with both trees and grasses colonized with endophytes. Thus we continue to work with tissues from the original set of *Populus trichocarpa* plants as well as from *Amelanchier alnifolia*, and *Lolium perenne* plants currently being greenhouse grown.

1. **Identify fungal endophyte infection status and remove systemic fungal endophytes from an important woody ornamental.**

To identify ubiquitous endophytes present in plant tissues, fungi will be identified directly from host tissues using common polymerase chain reaction method with a specific protocol developed by one of the authors (Hamilton and Faeth 2009).

UPDATE*:*

DNA was extracted from tissues (root and shoot) from hydroponically and pot grown plants to and a protocol has been optimized to identify endophytes *en planta* using a nested PCR protocol. New extraction and PCR methods were necessary for working with woody plants hosts and primers general to fungi rather than specific to *Neotyphodium.* Thus, the aforementioned protocol by Hamilton and Faeth (2009) could not be used.

2. **Determine if endophytic infection alters host response to generalist invertebrate herbivores and fungal pathogens.**

Generalist invertebrate herbivore treatments

A generalist invertebrate herbivore known to feed on *Populus* spp (e.g. gypsy moth caterpillars) will be collected from *Populus* in the field and reared on *Populus* tissue prior to experimentation to ensure their success on *Populus* (Frost et al. 2008). In a whole plant experiment 40 plants (20 per infection status) will be exposed to ten individual insects and net bagged as per Bultman et al. (2006).

Plants will be monitored for growth rate and biomass will be quantified for control versus insect infested plants. At two points during the experiment (before and after herbivory) plant tissue will be analyzed for total carbon (C), nitrogen (N) content. Herbivores will be evaluated prior to and after the feeding experiment for weight change and survival.

Fungal pathogen treatments

A ubiquitous fungal pathogen commonly infecting *Populus* spp. will be collected from *Populus* in the field. Leaf and stem tissue from E+ and E- hosts will be exposed to this fungus in *vitro*. Leaf tissue from 20 distinct plants (10 E+ and 10 E-) will be exposed to the pathogen and replicated five times. Growth of the fungal pathogen will be quantified by the size of the lesion formed.

Update:

We are collaborating with graduate students from Cornell's evolution and ecology (EEB) department for the use of two-spotted mites in herbivore experiments. Two generalist fungal pathogens (*Phoma medicaginis* and *Colletotrichum circinans*) and a Populus specific pathogen (*Marssonia populi*) have been isolated and cultured from host leaf tissues for use in pathogenic experiments. Following herbivore treatments, plants used in the original control group (32 trees) will be divided for exposure to one of the above mentioned plant pathogens (Figs. 1and 2) February 2011. Plant pathogens will be introduced to host leaves via one of two methods; 1) injection with a fine needle into 5 randomly selected leaf petioles, 2) wounding randomly selected leaves with insect pins followed by a foliar spray of inoculums at $1X10^{-8}$ strength.

3. Quantify changes in host biomass production as a general response by comparing E+ and E- controls.

The methods described in Objective #2 require the inclusion of controls, i.e. E+ and E- hosts not exposed to treatments. These hosts will provide the data to address this objective.

Results and discussion:

This research will produce data integral to the application of foliar endophytes as IPM agents by identifying the consequences of endophyte infection in terms of host response to various pests. The number of practitioners adopting endophytes as biological control agents is potentially industry-wide due to the ubiquity of symbionts and their successful application in other agronomic systems across a wide geographic range. We need only look to the use of mycorrhizal and rhizobial symbionts to imagine where foliar endophytes will take us in terms of integrated biological approaches. Application of mycorrhizal and rhizobial soil amendments is lest costly than pesticide and fertilizer applications with likely reduced damage to crops and humans. As demonstrated by our total crop loss following multiple pesticide applications, most plants especially forest trees have not been screened for their phytoxic responses. The used of generalist endophytes who have shared a long evolutionary history with various hosts, is much less likely to produce crop losses due to such phytoxic responses.

We are confident this research will contribute to the application of endophytes in IPM because endophytes have been successfully used for IPM in other plant systems (e.g. grasses). We are also confident this research can be completed in a timely manner due to the wealth of resources and knowledge available at Cornell's College of Agriculture and Life Sciences and affiliated communities.

The cost of implementing the research findings are confounded by the cost of getting a novel research program out of the greenhouse and into the field.

Resources developed:

We have successfully identified, mapped, and collected plant materials (branch, green wood cuttings) from 15 mature *Populus deltoides* trees (represents 15 unique genotypes) from Tompkins County area and rooted these in both hydroponics and soil-less mix. We have repeated this process with *Populus deltoides* with the same results. This demonstrates an effective greenhouse research protocol for testing the effects of fungicide applications on host trees. These methods will be part of a paper expected to be submitted in the spring of 2011.

We have inadvertently shown that both species of poplar are significantly and negatively impacted by broad spectrum insecticides. Greenhouse grown plants are susceptible to heavy two-spotted mite, thrip, and aphid infestations. This demonstrates the need to develop pesticides effective on tree species especially ones of important research and economic concern, e.g. *P trichocarp*.

Our use of fungicides as a means of removing fungal endophytes from shoot and root tissues of woody host trees has proved unsuccessful. We identified infections status of plants prior to fungicide applications and then took samples of tissues after two and four fungicide applications. Though not part of this research grant directly, these methods demonstrate two key considerations to working with fungal endophytes in tree species. First, there are plants from the field which appear to be uncolonized or as a result of overwintering followed by greenhouse germination become uncolonized. These can act as negative (E-) controls for comparisons with E+ plants without incorporating fungal endophytes. However, the confounding effects of genotype with colonization status can be problematic. As such we recommend that E- plants be cloned to allow for the opportunity to inoculate them with the endophyte(s) of interest to create a E-+ treatment (Figs. 1 and 2) which will control for genotypic effects . Second, fungicide applications to overwintered whips which leaf-out in the greenhouse are not effective in removing endophytes (or at least not the type of fungicide we used, Propiconazle 14.3 %; Table 1).

We have isolated numerous fungal endophytes from leaf tissues of *P. trichocarpa* and *A. alnifolia* and are maintaining pure isolate cultures in slants for long-term storage. These have been identified via sequencing (see Table 2) and will be part of the aforementioned publication. Multiple infections have been identified in addition to single isolates *en planta* (Table 2). We are currently optimizing a cloning protocol and cloning samples with multiple endophytes in order to identify individual members of the endophyte community present in single hosts (Image 2).

Work with a new grass host (*Lolium perenne*) harboring *Neotyphodium* endophytes (defensive mutualist) has been initiated. Though much research already documents decreased herbivory on endophyte colonized (E+) hosts little research has documented this endophyte's effect on

fungal plant pathogens. To determine *Neotyphodium's* effects on host's response to fungal pathogens the endophytes have been removed from a subset of seeds via standard heat treatments. We have germinated E+ and E- seeds in a greenhouse setting. We have isolated and cultured fungal pathogens for eventual pathogen treatments to E+ and E- seedlings. In February 2011 we will expose a subset of the grasses to two fungal pathogens and document: a) host survival, b) host biomass production, and c) pathogen lesion size.

Images, Figures, Tables

Herbivore	***Endophyte***		
	None (E-)	***Neo only (E+)***	***Both (E+-)***
Present	***8***	***8***	***8***
Absent	***8***	***8***	***8***

Figure 1. *A. alnifolia* plants of E+ and E- status to be used in herbivore experiments

Pathogen	***Endophyte***		
	None (E-)	***Neo only (E+)***	***Both (E+-)***
Phoma medicaginis	***4***	***4***	***4***
Colletotrichum circinans	***4***	***4***	***4***

Figure 2. Five randomly selected leaves from each plant will be inoculated with one of the plant pathogens originally isolated from the host. Following inoculation lesion development and various plant performance parameters will be measured to compare the effects of endophytes treatments on host's response to the fungal pathogens. These measures will include a) host survival, b) host biomass production, and c) pathogen lesion size.

Image 1. Roots produced on hydroponically treated *P. trichocarpa*.

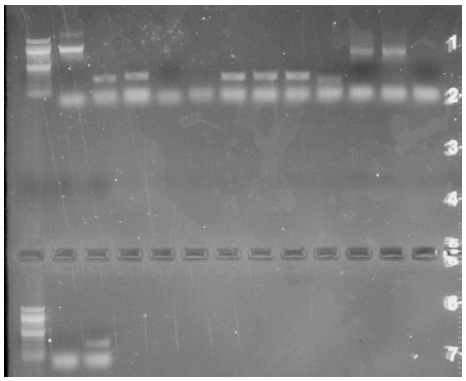

Image 2. Multiple bands are visible in the first, tenth, and eleventh lanes. No bands are present in lanes four, five, thirteen and fourteen (negative control); the remaining show single bands. The band pattern visible in all lanes is likely primer dimers.

	Fungicide	**No Fungicide**	**Chi-square**	**Probability**
E+ (colonized)	6	12	1	➢ 0.995
E- (not colonized)	7	5	0.16667	➢ 0.995

Table 1. Colonization frequencies of *Populus trichocarpa* exposed to fungicide either in soil soak application (95% of samples) or via hydroponic solution. Chi-square values given in terms of probabilities suggests no significant difference in E- frequencies in response to fungicide applications.

Plant Tissue	**Fungus**	**Fungal Life Strategy**
Populus trichocarpa; root and leaf	*Trametes versicolor*	Wood decaying, pathogen
Populus trichocarpa; leaf	*Xylaria* spp	Saprophyte, endophyte, pathogen
Populus trichocarpa; leaf	*Epicoccum* spp	Saprophyte, endophyte, pathogen
Populus trichocarpa; leaf	*Hymenochaete* spp	Wood decaying, pathogen
Populus trichocarpa; leaf	*Colletotrichum circinans*	Saprophyte, endophyte, pathogen
Populus trichocarpa; leaf	*Aspergillus niger*	Saprophyte
Populus trichocarpa; leaf	*Marssonia populi*	Pathogen
Populus trichocarpaI; root	*Fusarium poss graminearum*	Pathogen
Populus trichocarpaI; root	*Chaetomium* spp	Saprophyte, endophyte, pathogen
Populus trichocarpa; leaf	Unidentified endophyte from	Endophyte

	Sodariomycete	
Populus trichocarpa; leaf	Unidentified endophyte ECD2008	Endophyte
Populus trichocarpa; leaf	Basidiomycete	Saprophyte, endophyte, pathogen
Populus trichocarpaI; root	*Rhizoctonia* spp.	Saprophyte, pathogen
Populus trichocarpa; leaf	Uncultured endophyte 115-50	Endophyte
Populus trichocarpaI; root	*Bjerkandera* spp BOL13	Wood decaying, pathogen, saprophyte, endophyte
Amelanchier; root	*Trametes versicolor*	Wood decaying, pathogen
Amelanchier; root	*Colletotrichum circinans*	Saprophyte, endophyte, pathogen
Amelanchier; root	*Ischnoderma resinosum* strain CIRM-BRFM	Woody decay (white rot)

Table 2. Fungal identification determined via sequencing *en planta* or isolation from plant tissue and then sequencing from pure fungal cultures. Fungal life strategy determined via a review of the literatures and through the USDA ARS Plant-fungal database.